\TENT COOPERATION TRE. . Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 15 November 2000 (15.11.00)	
International application No. PCT/GB00/01118	**Applicant's or agent's file reference** P1221/WOD
International filing date (day/month/year) 23 March 2000 (23.03.00)	**Priority date** (day/month/year) 24 March 1999 (24.03.99)
Applicant ALPAR, Hazire, Oya et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 October 2000 (12.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Juan Cruz Telephone No.: (41-22) 338.83.38

GB0001118

Form PCT/IB/331 (July 1992)

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2
MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
24 July 2001 (24.07.01)

IMPORTANT NOTIFICATION

Applicant's or agent's file reference
P1221/WOD

International application No.
PCT/GB00/01118

International filing date (day/month/year)
23 March 2000 (23.03.00)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
BOWDERY, A., O.
D/IPR
Formalities Section
Poplar 2
MOD Abbey Wood #19
Bristol BS34 8JH
United Kingdom

State of Nationality:
State of Residence:
Telephone No.: 0117 91 32860
Facsimile No.: 0117 91 32930
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2
MOD Abbey Wood #19
Bristol BS34 8JH
United Kingdom

State of Nationality:
State of Residence:
Telephone No.: 0117 91 32860
Facsimile No.: 0117 91 32930
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
R. Chrem

Telephone No.: (41-22) 338.83.38

004170317

Form PCT/IB/306 (March 1994)

I ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2
MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
24 July 2001 (24.07.01)

Applicant's or agent's file reference
P1221/WOD

International application No.
PCT/GB00/01118

IMPORTANT NOTIFICATION

International filing date (day/month/year)
23 March 2000 (23.03.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
THE SECRETARY OF STATE FOR DEFENCE
CBD Porton Down
Salisbury
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
THE SECRETARY OF STATE FOR DEFENCE
DSTL
Porton Down
Salisbury
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
R. Chrem
Telephone No.: (41-22) 338.83.38

004170318

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

BOWDERY, A., O.
D/IPR
Formalities Section
Poplar 2
MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
09 July 2001 (09.07.01)

Applicant's or agent's file reference
P1221/WOD

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/01118

International filing date (day/month/year)
23 March 2000 (23.03.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

THE SECRETARY OF STATE FOR DEFENCE
Defence Evaluation and Research
Agency
Ively Road
Farnborough
Hampshire GU14 0LX
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address

THE SECRETARY OF STATE FOR DEFENCE
CBD Porton Down
Salisbury
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
R. Chrem

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P1221/WOD		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/01118	23/03/2000	24/03/1999
International Patent Classification (IPC) or national classification and IPC A61K39/39		
Applicant THE SECRETARY OF STATE FOR DEFENCE et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/10/2000	10.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Moreno de Vega, C Telephone No. +49 89 2399 7486

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/01118

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-22	as originally filed				

Claims, No.:

1-35	as received on	23/02/2001	with letter of	19/02/2001	

Drawings, sheets:

1/6-6/6	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 30-34.

because:

   ☒ the said international application, or the said claims Nos. 30-34 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

   Novelty (N) Yes: Claims 1-6, 13-15, 17, 19-29

	No:	Claims	7-12, 16, 18, 30-35
Inventive step (IS)	Yes:	Claims	23-29
	No:	Claims	1-22, 30-35
Industrial applicability (IA)	Yes:	Claims	1-29, 35
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 30-34 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: WO 97 20576 A (DANBIOSYST) 12 June 1997 (1997-06-12)
D2: WO 96 10421 A (MEDEVA HOLDINGS) 11 April 1996 (1996-04-11)
D3: US-A-5 585 106 (GRISTINA A.G. ET AL.) 17 December 1996 (1996-12-17)

This International Examining Authority has considered the applicant's arguments in response to the Written Opinion and is of the following opinion:

1. Novelty (Article 33(2) PCT)

D1 (see claims) discloses a vaccine composition for intranasal administration, which comprises antigens and an effective adjuvant amount of chitosan, formulated as microspheres, and methods of enhancing an immune response and of immunizing hosts using said composition. This document appears to be novelty destroying for claims 7-12, 16, 18, 30-35.

D2 (see claims) describes vaccine compositions adapted for mucosal administration, comprising influenza virus antigen and an effective adjuvant amount of chitosan, and methods for enhancing the immunoresponse to influenza virus antigens and of immunising a host using said compositions.

This document appears to be novelty destroying for claims 7-12, 18, 30-35.

Thus, claims 7-12, 16, 18 and 30-35 do not meet the requirements of Article 33(2) PCT.

2. Inventive step (Article 33(3) PCT)

Present claims 1-6 differ from D1, which is considered to be the most relevant prior art, in the use of an alkylated chitosan instead of others chitosans in a polycationic carbohydrate immunostimulant. The technical problem to be solved by claims 1-6 is the provision of a immunostimulant. This problem was solved in D1 by the use of chitosans in the preparation of vaccines. It appears that the use of specific chitosans in these claims is a mere selection of the possible known chitosans, as depicted in the description. Thus, claims 1-6 are not considered to be inventive.

Present claims 13-15, 17, 19 and 20-22 differ from D1, which is considered to be the most relevant prior art, in that polymeric materials like poly-L-lactide are used to form particles of antigen, that the antigens used are Y. pestis antigens, in the use of the composition for parenteral administration, and the addition to the compositions of further compounds like a cationic pluronic. The technical problem to be solved by these claims is the provision of compounds useful as immunostimulants and compositions containing them for the preparation of improved vaccines.

The use of poly-L-lactide as a particle forming material in vaccines is already known in the art (D3). Thus, claim 14 is not considered to be inventive.

Claims 13, 15, 17 and 19 do not contain any additional technical feature which could be considered to involve an inventive step in the light of the disclosure in D1 and D2 and the common practice in the field.

The use of cationic pluronics as surfactants, emulsion-formers and vehicles in the formulation of pharmaceutical compositions and vaccines is already known for the person skilled in the art, who would have tried them to improve

the adjuvant compositions of D1 and D2 without exerting an inventive activity. Therefore, claims 20-22 are not considered to be inventive.

Claims 23-29 differ from D1 and D2 in the methods for producing pharmaceutical compositions as featured in these claims. The technical problem to be solved by claims 23-29 is the provision of methods for preparing pharmaceutical compositions with improved immunostimulant properties. The methods of these claims are neither disclosed nor suggested in the known prior art. Claims 23-29 are therefore considered to be inventive.

3. For the assessment of the present claims 30-34 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VII

Certain defects in the international application

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 and D2 is not mentioned in the description, nor are these documents identified therein.

From the
INTERNATIONAL PRELIMINAR[redacted]AMINING AUTHORITY

To:

SKELTON, S. R.
D/IPR
Formalities Section (DERA)
Poplar 2, MOD Abbey Wood 19
Bristol BS34 8JH
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	10.05.2001
Applicant's or agent's file reference: P1221/WOD	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/01118	23/03/2000	24/03/1999

Applicant: THE SECRETARY OF STATE FOR DEFENCE et al

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

   The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

   Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

   For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Neumann, M Tel.+49 89 2399-7351

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P1221/WOD		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/01118	23/03/2000	24/03/1999
Applicant		
THE SECRETARY OF STATE FOR DEFENCE		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   POLYCATIONIC CARBOHYDRATES AS IMMUNOSTIMULANTS IN VACCINES

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 39/39, 39/02, 39/05, 9/127, 9/50, A61P 31/04

A2

(11) International Publication Number: WO 00/56362

(43) International Publication Date: 28 September 2000 (28.09.00)

(21) International Application Number: PCT/GB00/01118

(22) International Filing Date: 23 March 2000 (23.03.00)

(30) Priority Data:

9906696.1	24 March 1999 (24.03.99)	GB
9906694.6	24 March 1999 (24.03.99)	GB

(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation and Research Agency, Ively Road, Farnborough, Hampshire GU14 0LX (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: ALPAR, Hazire, Oya [GB/GB]; Aston University, Aston Triangle, Birmingham B4 7ET (GB). EYLES, James, Edward [GB/GB]; Aston University, Aston Triangle, Birmingham B4 7ET (GB). SOMAVARAPU, Satyanarayana [IN/GB]; Aston University, Aston Triangle, Birmingham B4 7ET (GB). WILLIAMSON, Ethel, Diane [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). BAILLIE, Leslie, William, James [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agent: BOWDERY, A., O.; D/IPR, Formalities Section, Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: IMMUNOSTIMULANTS

(57) Abstract

A polycationic carbohydrate such as chitosan, or a pharmaceutically acceptable derivative thereof, are used as immunostimulants. Vaccine compositions containing these polycationic carbohydrates, in particular in particles such as microparticles or liposomes are also described and claimed. Methods of treatment and the use of the polycationic carbohydrates as immunostimulants in the production of vaccines are further aspects described and claimed.

FOR THE PURPOSES OF INFORMATION ONLY

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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Immunostimulants

The present invention relates to compounds which are polycationic carbohydrates, for use as immunostimulants and to vaccines containing these. The invention further comprises methods of treating individuals using the pharmaceutical compositions containing the compounds as well as methods of preparing the compositions.

A prime objective in the field of vaccination is the development of a non-parenteral immunisation regimen, which facilitates induction of comparable levels of systemic immunity to that elicited by conventional sub-cutaneous and intra-muscular injections.

The nasopharyngeal passages and pulmonary regions of the respiratory tract represent potential targets for the systemic delivery of peptidergic drugs and vaccines. The relative ease with which therapeutic agents can be inhaled, or introduced into the nose, make these modes of immunisation attractive in terms of probable patient compliance. Furthermore, respiratory mucosae offer certain morphological, physiological and immunological advantages over other non-parenteral sites in terms of immunisation, particularly against pathogenic entities which affect or utilise mucosal surfaces as portals of entry. This is because effective vaccination against these pathogens normally requires mucosae to be adequately protected with locally produced antibodies of the secretory IgA (sIgA) isotype. Whilst mucosal surfaces are usually poorly protected with IgA following parenteral administration of vaccines, it is now apparent that successful delivery of antigenic material to immunoresponsive elements in mucosa-associated lymphoid tissue (MALT) can result in vigorous stimulation of the mucosal arm of the immune system. By means of the common mucosal immune system (CMIS) it is feasible that several anatomically disparate mucosal surfaces could be protected through mucosal

administration of a vaccine at a single site. Mucosal vaccination offers the added advantage that some degree of systemic immunity can be induced in concert with local responses due to translocation of antigenic material from sub-epithelial compartments to systemic immunoresponsive tissues such as the spleen.

Despite the logistical and immunological factors which favour non-parenteral immunisation, simple mucosal application of antigenic proteins, for example in the gastrointestinal or respiratory tracts, is usually ineffectual in terms of vaccination. Enzymatic or chemical destruction, combined with poor absorption into sub-epithelial compartments dictate that mucosally administered vaccines usually require some form of adjuvant or delivery vehicle. One approach is to encapsulate antigenic material within microparticulate polymeric carriers, such as poly-DL-lactide (PLA) microspheres (Vaccine 1994, 12, 5-11). Such procedures serve to protect labile vaccines from lumenal degradation and enhance absorption into mucosal and systemic compartments (J.H. Eldridge et al., Seminars in Hematology, (1993), 30, 16-25). There is good evidence that microencapsulation may also adjuvantise by converting soluble antigenic molecules into particulate species, thus promoting vaccine uptake into antigen presenting cells (APC)(Y. Tabata et al., Adv. Polym. Sci. (1990), 94, 107-141, L. Vidard et al., J. Immunol. (1996), 156, 2809-2818, N. Van Rooijen, Immunol. Today (1990) 11, 436-439) or microfold cells (M-cells) in lymphoid follicles (R.I. Walker et al., Vaccine, 12, 387, 1994, D.T. O'Hagan et al,, Vaccine, 1989, 7, 421-424, P.G. Jenkins et al., J. Drug Targetting, 1995, 3, 79-81).

Although comparatively under-investigated, the intra-nasal (i.n.) route is an attractive one for the mucosal delivery of vaccinal entities. The nasal epithelium is accessible and is less exclusive to high molecular weight molecules.

The thickness of the mucus blanket covering respiratory epithelium is relatively thin compared to that of other mucosae, for example the gut where it is in the region of 500 times thicker. Substantially reduced concentrations of proteolytic enzymes and extremes of pH exist in the respiratory tract compared with the gastrointestinal tract.

Furthermore, it is now delineated that nasal associated lymphoids tissues (NALT) have a lymphoepithelium which, like that in the intestinal mucosa, contain M-cells for selective antigen uptake (P. Brandenburg, Immunology of the Lung and Upper Respiratory Tract, (ed. Bienenstock J.) McGraw-Hill, New York, 1984, 28-95). Hence NALT plays an analogous role to other MALT, such as the gut associated lymphoid tissues (GALT), in terms of antigen surveillance and induction of mucosal and systemic immunological responses.

Polycationic carbohydrates such as chitosan as well as various derivatives or composites of these proteins have been used previously in the pharmaceutical compositions as absorption enhancers to increase delivery of an active pharmaceutical agent across a barrier such as a mucosal membrane. The applicants have found however, that when these compounds are used in the context of vaccine compositions, they act as adjuvants, producing an increase in the immune response to the antigenic agent being delivered. The increase in the response levels persists for considerably longer than would be expected as a result only of absorption enhancing effects.

Thus according to the present invention there is provided a polycationic carbohydrate or a pharmaceutically acceptable derivative thereof, for use as an immunostimulant.

As used herein, the term "immunostimulant" refers to an adjuvant which stimulates the immune system of a host animal to which it is administered and thereby increases the protective effect

produced by a protective antigen administered to that animal, as compared to the effect which would be produced by administration of the protective antigen alone.

The expression "polycationic carbohydrate" includes polymeric compounds with repeat units of general formula $C_x(H_2O)_y$. They will include multiple cationic functions or group such as amino or amido groups which are cationic in nature in that they will readily form cations such as quaternary ammonium compounds.

Particular examples of polycationic carbohydrates include immunostimulant chitin derivatives such as chitosans or chemically modified forms such as soluble forms thereof; cationic polypeptides; cationic polyamino acids; and quaternary ammonium compounds; or mixtures thereof.

Examples of water-soluble derivatives of polycationic carbohydrates in particular, are water-soluble chitin derivatives such as an alkylated chitosan derivatives and salts thereof.

Examples of these chemicals include trimethyl chitosan chloride, carboxymethyl chitosan, N-carboxymethyl chitosan, and polyethylene glycol chitosan. N-trimethyl chitosan chloride (TMC) has been referred (Kotze, A. F. et al. Pharm Res. (1997) 14:1197-1202) as a potential absorption enhancer of peptide therapies across mucosal membranes. In contrast to chitosan, TMC is water soluble in all gastrointestinal pH environments. Further, it retains the ability to temporarily open tight junctions. An important parameter appears to be the degree of trimethylation.

Other examples of chitosan derivatives which might be used in the context of the invention include chitosan chloride and chitosanglutamate.

Examples of biologically active agents which are capable of generating a protective immune response in an animal, particularly a mammal, and which may benefit from combined use with the immunostimulants of the invention are well known in the art. They include antigenic polypeptides as well as nucleic acid sequences which may encode these polypeptides and which are known as "naked DNA" vaccines. Live vaccines such as DNA or RNA viral vaccines may also be used in conjunction with the immunostimulant of the invention.

As used herein the expression "polypeptide" encompasses proteins or epitopic fragments thereof.

Suitable polypeptides are sub-unit vaccines, such as tetanus toxoid, diptheria toxoid and *Bacillus anthracis* protective antigen (PA).

A particular example of a biologically active agent is one which is capable of generating a protective immune response against *Yersinia pestis*. The agent is suitably a sub-unit vaccine, for example as described in WO 96/28551. The vaccine described and claimed there comprises a combination of the V antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these, and the F1 antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these.

As used herein, the term "fragment" refers to a portion of the basic sequence which includes at least one antigenic determinant. These may be deletion mutants. One or more epitopic region of the sequence may be joined together.

The expression "variant" refers to sequences of nucleic acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced

with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% homologous, preferably at least 75% homologous, and more preferably at least 90% homologous to the base sequence. Homology in this instance can be judged for example using the algorithm of Lipman-Pearson, with Ktuple:2, gap penalty:4, Gap Length Penalty:12, standard PAM scoring matrix (Lipman, D.J. and Pearson, W.R., Rapid and Sensitive Protein Similarity Searches, *Science*, 1985, vol. 227, 1435-1441).

Previously, we have demonstrated that the intranasal (IN) route is a highly effective, non-invasive alternative, to the parenteral administration of recombinant subunit vaccines (F1 and V derived from the causative organism of plague: *Yersinia pestis*) (Eyles, J. E. et al. Vaccine (1998) 16:698-707; J. Drug Targeting Nasal delivery of vaccines) 1996 Alpar & Almeida). Fraction 1 (F1) subunit (molecular mass 15.5 kDa) is derived from the capsule that surrounds the bacteria. In solution, because of its hydrophobic nature, F1 tends to aggregate into multimeric complexes of high (>200,000 kDa) molecular weight (Voronisov, E. D. et al. Biomed. Sci. (1990) 1:391-396 and Miller, J. et al. FEMS Immun. Med. Micro. (1998) 21:213-231). The V antigen (molecular mass 37 kDa) is a protein secreted by the bacterium at 37°C (Leary, S. E. C. et al. Infect. Immun. (1995) 63:2854-2858). V is a virulence factor which may exert local anti-inflammatory effects through modulation of tissue cytokine levels (Nakajima, R. and R. R. Brubaker Infect. Immun. (1993) 61:23-31). Both F1 and V are protective, and there is a documented 'synergistic effect' in combination (Williamson, E. D. et al. Vaccine (1996) 14:1613-1619). Traditionally killed whole cell vaccines for plague have an unsatisfactory incidence of transient local and systemic side effects, but more

importantly, may fail to protect individuals from the pneumonic form of the disesase, which is transmissible *via* airborne droplets (Perry, R. D. and J. D. Fetherston. Clin. Microbiol. Rev. (1997) 10:35-66). Intramuscular injection (Williamson, E. D. et al. Vaccine (1997) 15:1079-1084) of combined F1 and V, or intranasal administration of microspheres co-encapsulating F1 and V (Eyles, J. E. et al. Vaccine (1998) 16:698-707), can protect experimental animals against a lethal inhalational challenge with *Y. pestis*.

Following these experiments it was seen that systemic immunity, in the form of high serum IgG titres to F1 and V, was critical for protection. In the current work, the applicants have nasally instilled F1 (5 μg) and V (1 μg) in the presence and absence of a variety of chemicals including three different TMC derivatives (with increasing degrees of quaternization: 20, 40 and 60%), and compared humoral immune response engendered by these treatments to those evoked by co-administration of chitosan itself. The results indicate that the high the degree of derivitisation, the greater the increase in the immune response. Thus TMC 60 is the most preferred form.

In a further aspect, the invention provides a method of protecting an animal against a pathogen, said method comprising administering to said animal, a protective agent which is able to stimulate the animal's immune system to produce a response which is protective against said pathogen, and an immunostimulant comprising a polycationic carbohydrate.

Suitable animals are mammals including humans.

The adjuvant or immunostimulant may be administered simultaneously with said protective agent, suitably in a combined formulation. Alternatively, it may be administered separately. The selection of particular administration conditions will vary depending upon factors such as the nature

of the protective agent, the condition being treated, the age and size of the animal etc.

Preferably however the immunostimulant of the invention will be administered to the animal in a single pharmaceutical composition with the protective agent.

Suitably the composition will further comprise a pharmaceutically acceptable carrier or diluent. These may be solid or liquid carriers as are known in the art. Carriers or diluents may vary depending upon the particular nature of the protective agent and the particular polycationic carbohydrate used. They may comprise pharmaceutically acceptable solvents such as water in which the protective agent and the polycationic carbohydrate are dissolved. This type of formulation is particularly suitable when the protective agent is itself water-soluble.

Compositions in the form of solutions of this type suitably contain from 0.1 to 30% w/v and preferably from 1 to 20% w/v of polycationic carbohydrate, depending upon its solubility.

The composition is suitably adapted for parenteral administration such as intramuscular (i.m.) administration, or it may be suitable for administration to mucosal surfaces of the animal.

Administration to mucosal surfaces may be effected by oral application, by pulmonary application, for example by intra-tracheal administration, or particularly by intra-nasal application. In particular, the compositions of the invention are administered by the intra-nasal route.

For many applications however, it has been found preferable that the protective agent and the polycationic carbohydrate immunostimulant is microencapsulated in a polymeric material and

thus the carrier is a particulate carrier such as a microsphere or microparticle (also known as a microcapsule), nanocapsule or liposome.

In a preferred embodiment, a polycationic carbohydrate which is able to act as an immunostimulant is provided in the form of a pharmaceutical composition, which composition comprises particles comprising

(i) a biologically active agent which is able to produce an immune response in an animal to which it is administered;

(ii) a first material capable of forming particles; and

(iii) said polycationic carbohydrate.

The particles are suitably incorporated into a composition which is adapted for administration to mucosal surfaces. In addition, the particles may suitably be incorporated into a composition which is adapted for administration using a parenteral route such as i.m. injection.

Suitably the particles comprise microparticles or microspheres or liposomes.

The first material used in the compositions of the invention is suitable for forming microspheres or liposomes. Liposome production requires the use of lipids and/or surfactant type molecules as is understood in the art.

Preferably however, the composition of the invention comprises a microsphere. In this case the first material comprises a polymer. It may be a low, medium or high molecular weight polymer. Examples of low molecular weight polymers are polymers which have a molecular weight of between 0.1 and 10kDa, more preferably between 1 and 5 kDa and typically about 2-3kDA.

The use of high molecular weight polymers in the encapsulation of a tetanus vaccine for intramuscular administration has been

described (Vaccine 1994, 12, 4, 299-306). A formulation of microencapsulated ricin toxoid vaccine which is applied intranasally has also been described (Vaccine 1994, 14, 11 1031). However, in that case, high molecular weight polymer microparticles (94kDa) were less effective than those prepared from a copolymer of lower molecular weight (72kDa).

The polymeric material used as the first material in the composition of the present invention suitably has a high molecular weight in excess of 94kDa, for example of 100kDa or more.

A particularly suitable polymeric first material for use in the compositions of the invention comprises poly-(L-lactide) or PLA but other high molecular weight polymeric material such as poly(lactic/glycolic acid) PGLA, polycyanacrylates, polyanhydrides or polycaprotactones as are known in the art may be employed.

Suitably the polycationic carbohydrate is present in the composition in an amount of from 0.1% to 10%w/w. Particular examples of suitable polycationic carbohydrates for use in the above described particle compositions include chitin derivatives such as chitosans; cationic polypeptides; cationic polyamino acids; and quaternary ammonium compounds; or mixtures thereof.

The compositions may optionally further comprise agents which stabilise emulsions such as polyvinylalcohol or methyl cellulose.

They will suitably be of an average size of from 0.1μm to 10μm in diameter.

Optionally, vaccine compositions may further comprise an additional conventional adjuvant in order to enhance the immune response to the biologically active material administered.

Suitable adjuvants include pharmaceutically acceptable adjuvants such as Freund's incomplete adjuvant, aluminium compounds and, preferably adjuvants which are known to up-regulate mucosal responses such as CTB, the non-toxic pentameric B subunit of cholera toxin (CT).

Yet further adjuvants are described and claimed in the applicants copending International application of even date, derived from British Patent Application Nos. 9906694.6 and 9906696.1. In particular these compounds are

A) a polyamino acid,
B) a vitamin or vitamin derivative,
C) cationic pluronics,
D) a clathrate,
E) a complexing agent,
F) cetrimides;
G) an S-layer protein; or
H) methyl-glucamine.

As used herein, the expression "cationic pluronics" includes both pluronics which include cations, as well as those which have been treated such that they are bound to cationic moieties (cationised pluronics).

Suitable biological agents (i) include drugs and therapeutic molecules such as vaccines, antivirals, antibiotics, antifungals, antiparasitics as well as oligonucleotides used in therapies and vaccines.

However in a preferred embodiment, the biologically active agent is an agent that is capable of generating an immune response in an animal to which it is administered and most preferably a protective immune response. Thus the compositions are suitably used as vaccines including those which rely on oligonucleotides or other nucleic acid sequences. In this case, the immunostimulant properties of the compounds A-H are used.

Suitably the said adjuvant chemical is soluble in water. Suitably the composition is suitable for non-parenteral administration for example to mucosal surfaces or for topical application to the skin. Particularly preferred compositions are suitable for administration to mucosal surfaces.

Alternatively, the composition is suitable for parenteral administration for example by intramuscular (i.m.) administration.

Administration to mucosal surfaces may be effected by oral application, by pulmonary application, for example by intra-tracheal administration, or particularly by intra-nasal application. In particular, the compositions of the invention are administered by the intra-nasal route.

Examples of adjuvant chemicals in category (A) above include polyamino acids such poly-ornithine, for example of molecular weight from 5 to 150kDa.

Particular examples of adjuvant chemicals in category (B) above are vitamins or vitamin derivatives such as vitamin E or derivatives for example vitamin E TPGS (d-alpha tocopheryl polyethylene glycol 1000 succinate).

Particular cationic pluronics in category (C) above, are block copolymers or surfactants which are positively charged, in particular with NH_2^+ groups. These are available commerically for example from ICI Ltd (UK) sold under the trade names P101 and P121.

Examples of clathrates in category (D) above include in particular cyclodextrins and their derivatives such as dimethyl β cyclodextrin.

Suitable complexing agents in category (E) above are bile salts, in particular those which form complexes with fatty acids such as deoxycholic acid.

Examples of cetrimides in category (F) are quaternary ammonium compounds used as preservatives.

In particular, the compositions of the invention may further comprise a cationic pluronic as described above. These may be included either within the particles, or as a component of the dosing mixture or both. In a particularly preferred embodiment, the cationic pluronic is formed into microparticles and in particular nanospheres, which are then coated with the polycationic carbohydrate such as chitosan. Biologically active material may then be absorbed onto the coated particles.

They may also comprise other known composition components such as colouring agents and preservatives and in particular cetrimide. These are suitably present in amounts of from 0.1 to 0.7%w/v.

In a particular embodiment, the microspheres or liposomes used in the compositions may further comprise a S-layer proteins, in particular, S-layer proteins derived from a bacteria against which the biologically active agent produces a protective immune response. These may be distributed throughout the microspheres or liposomes but are preferably coated on the surface. It has been shown (Sleyr et al., Crystalline bacterial cell surface proteins. Biotechnology Intelligence Unit, 1996, R.G. Landes Company and Academic Press Inc.) that the stability of liposomes can be increased by such coatings. S-layer proteins are found on the surface of most bacteria and form a regular two dimensional array known as an S-layer. Isolated S-layer proteins are able to form entropy driven monomolecular arrays in suspension, and on the surface of structures such as liposomes.

The above-described particle composition is particularly suitable for intranasal application. They may comprise particles such as microparticles per se which are optionally preserved, for example by lyophilisation, or the microparticles may be combined with a pharmaceutically acceptable carrier or excipient. Examples of suitable carriers include solid or liquid carriers as is understood in the art.

The invention further provides a method of producing a pharmaceutical therapeutic vaccine, which method comprises encapsulating a biologically active agent as described above in a first polymeric material which has a high molecular weight and in particular a molecular weight of 100kDa or more, in the presence of a polycationic carbohydrate such as chitosan. The polycationic carbohydrate may be incorporated within the microparticle, or at the surface, or preferably is distributed throughout the microparticle including at the surface.

In some cases, particularly where the polycationic carbohydrate is insoluble, it may preferably be adsorbed onto the surface of a pre-formed particle. It is possible also to load biologically active agent (such as a protective antigen) onto the thus formed particles.

Methods of forming liposomes are well known in the art. They include dispersion of dehydrated lipid films into aqueous media, emulsion techniques and lyophilisation methods as are well known in the art.

Microparticles of the invention are suitably prepared using a double emulsion solvent evaporation method. Briefly, the biologically active agent, suitably in a lyophilised state, is suspended in an aqueous solution of the first polymer such as polyvinyl alcohol (PVA) and the polycationic carbohydrate such as chitosan. A solution of the high molecular weight polymer in an organic solvent such as dichloromethane, is added with

vigorous mixing. The resultant emulsion is then dropped into a secondary aqueous phase, optionally containing polycationic carbohydrate, with vigorous stirring. After addition, the organic solvent is allowed to evaporate off and the resultant microspheres separated.

Preferred biologically active agents and first materials and polycationic carbohydrates are as described above.

The compositions will suitably comprise an appropriate dosage unit of the active agent. This will vary depending upon the nature of the active agent being employed, the nature of the patient, the condition being treated and other clinical factors. In general however, the composition of the invention will comprise approximately 2 to 10 wt% of active ingredient.

In microparticle containing compositions of the invention the amount of first material, in particular the high molecular weight polymer, in the composition will be of the order of 70 to 99wt% of the composition, and suitably from 90 to 99wt% of the polymer components will be the first polymer. The amount of polycationic carbohydrate material, such as chitosan or a mixture of chitosan with other positively charged molecules, will be of the order of 0.1 to 10 wt % of the composition.

-In use, a reasonable dosage for nasal administration would be of the order of 0.05g.

Thus, in a further aspect, the invention provides a method of protecting a mammal against infection, which method comprises administration of a vaccine composition as described above to a mucosal surface, in particular a nasal surface, of a mammal.

The applicants have demonstrated that it is possible to protect experimental animals from inhalation challenge with *Y. pestis* through i.n. administration of a combined sub-unit vaccine. The

adjuvantisation of these sub-units is advantageous in enhancing the immune response as is microencapsulation of the sub-units. The high molecular weight polymer utilised in the compositions of the invention appears to be particularly well suited to intra-nasal delivery.

Alternatively, however, the immunostimulants of the invention may be administered as i.m. formulations and long term beneficial effects are still noted.

The invention will now be particularly described by way of example with reference to the accompanying drawings in which:

Figure 1 illustrates the serum immune response in mice to nasally delivered microencapsulated and free diptheria toxoid with 10 lf units on day 1 and day 67, where the first column represents results with microsphere without chitosan, the second column represents the results of microspheres with chitosan and the third column shows the results with free diptheria toxoid alone;

Figure 2 illustrates the specific serum antibody responses following a single nasal application of 1µg V and 5µg F1 antigens of *Yersinia pestis* in compositions according to the invention; and

Figure 3 shows the specific serum antibody responses over a period up to 86 days following intramuscular injection of free of microencapsulated BSA in the presence or absence of chitosan;

Figure 4 shows the serum immune response to nasally delivered Diptheria toxoid (DT) adsorbed onto nanoparticles whose surface has been modified cationic with chitosan derivatives;

Figure 5 shows the results of photon correlation spectroscopy (PCS) on particles produced for use in the invention, showing

that the number mean diameter (d_n) and the volume mean diameter (d_v) were both around 150nm; and

Figure 6 illustrates the immune response to Diptheria toxoid (DT) in various formulations applied by i.m. routes, where P101 is a pluronic 101 and P121 is a block co-polymer available from ICI Ltd, UK.

Example 1

Microencapsulation of diptheria toxoid

Poly-L-lactide of molecular weight 100kDa (Polysciences Inc. USA) was used in a modification of the double emulsion solvent evaporation method (Y. Ogawa et al., Chem. Pharm. Bull., 36 (1988) 1095-1103). Briefly, 1.5ml of a 0.75% w/v of chitosan solution containing diptheria toxoid was vigorously mixed with 200mg of 100K PLA polymer dissolved in 5ml of HPLC grade dichloromethane (DCM) using a Silverson homogeniser (Silverson, UK) for 1 minute. The resultant primary emulsion was added, drop by drop, into a secondary aqueous phase (75ml) containing 0.5%w/v chitosan and homogenised using a Silverson homogeniser for 5 minutes. This secondary phase was gently stirred overnight until the dichloromethane had evaporated. Microspheres were recovered by centrifugation, washed with double distilled water three times and then lyophilised.

Example 2

Immunisation Study

Balb/c female mice (25g, 6-week old) were lightly anaesthetised using an inhaled gaseous mixture of 3% halomethane (RMB Animal Health Ltd., UK) in oxygen ($300cm^3\ min^{-1}$) and nitrous oxide ($100cm^3min^{-1}$) for intranasal dosing procedures. Groups of mice received one of the following treatments:

(1) Microspheres prepared as described in Example 1 but in the absence of chitosan;

(2) Microspheres prepared as described in Example 1; and

(3) Free diptheria toxoid solution.

Each were administered in 50µl of PBS using a micropipette. Groups of mice each received 10 lf units on day 1 and day 67 of either microencapsulated or free diptheria toxoid.

Serum immune responses were monitored. Tail vein blood samples were taken from all animals on days 14, 28, 95 and 151 of the experiment. Titration of IgG and IgA antibody isotypes in serum samples was achieved using an ELISA. Briefly, individual serum samples were aliquoted to microtitre plates pre-coated with diptheria toxoid. Binding of serum antibody was detected with peroxidase-labelled secondary antibody to mouse IgG (Sigma A4416) or AgA (Sigma A4789). Antibody titre was estimated as the maximum dilution of the serum giving an absorbance reading greater that the maximum optical density (OD) of titrated naïve serum. From this, mean titres $\pm$ standard deviation (SD) were derived per treatment group.

The results are shown in Figure 1. Throughout the 151 day schedule, mice dosed with microencapsulated antigen in the absence of chitosan (Group 1) maintained statistically elevated serum IgG titres to diptheria toxoid in comparison to animals treated with free vaccine (Group 3) (Figure 1). However, the levels of IgG titres to diptheria toxoid in Group 2 animals was consistently higher still indicating that the presence of chitosan in the microparticle enhances the immune response to the toxoid.

Example 3

Use of absorption enhancers to enhance immunological response to intranasally administered subunit vaccines

Five groups of five (n=5) BALB/c mice were intranasally immunised with admixed F1 (5 µg) and V (1 µg). The five treatment groups received the subunits in conjunction with either: 1) Phosphate buffered saline (pH 7.4); 2) 0.2% w/v

chitosan HCL; 3) 0.2% w/v TMC 60; (4) 0.2% w/v TMC 40; (5) 0.2% w/v TMC 20. A further group of animals acted as a control.

Mice were lightly anaesthetised with an inhaled gaseous mixture of 3% (v/v) halothane (RMB Animal Health Ltd., UK) in oxygen ($300cm^3\ min^{-1}$) and nitrous oxide ($100cm^3\ min^{-1}$) for i.n. dosing procedures. Each mouse received a 15 µl volume of liquid administered with a micropipette. Tail vein blood samples were taken on day 14, and serum was analysed for the presence of anti-V and anti-F1 IgG antibodies using an indirect ELISA protocol (Eyles, J. E. et al. Vaccine (1998) 16:698-707).

The results (Figure 2) indicate that mucosal co-administration of TMC60 or TMC40 augments the humoral response to F1 and V above and beyond that generated by i.n. instillation of F1 and V in phosphate buffered saline or chitosan HCl. TMC20 failed to improve titre to V, although the effect on immunity to F1 was comparable with that of the more substituted chitosan derivatives (TMC40 & 60).

Example 4

Intramuscular administration of immunostimulants.

Microspheres incorporating bovine serum albumin (BSA) were prepared from poly-L-lactic acid of molecular weights 2kD, 50kD or 100kD (or a combination of these) in the presence or absence of chitosans using a double emulsion method (dichloromethane/water). These preparations were administered to mice in a single intramuscular dose in a 50µl volume of sterile PBS ('Day 0' of the study, 15µg BSA equivalent, typically 1mg microspheres). Blood samples were taken from mice at a number of subsequent timepoints and the serum analysed by ELISA for anti-BSA IgG levels.

The results are shown in Figure 3. In this graph, the following key applies:

M= intramuscular;
F = free BSA;
E5 = BSA encapsulated into microspheres composed from a 1:1 mass ratio of 2kD:100kD PLA;
LC = low MW chitosan.

Surprisingly it was found that free antigen plus microencapsulated BSA in the presence of chitosan performed better in vivo than other dosing administrations containing only one of these components with free antigen, or free antigen administered alone.

Example 5

Surface modification using chitosans

500mg of PDLA (124kD) was dissolved in dichloromethane (DCM). To this solution, an aqueous solution of 2.5%(w/v) polyvinylalcohol (PVA) was added while probe-sonicated. The resultant primary emulsion was added to 20ml of a colled aqueous solution of 1.5% (w/v) PVA under homogenisation (Silverson SL-2 homogeniser), to form a secondary emulsion. A further 10ml of water was added to the resulting emulsion and the formulation stirred overnight. Particles were washed and collected by centrifugation with distilled water at 3x20 min at 15,000rpm, before finally being freeze-dried.

Four batches of particles were prepared as described above, three of which were surface-modifed, either with N-carboxymethyl chitosan (Canada), chitosan chloride or chitosan glutamate (Pronovo, Norway) by adding 10mg of the relevant chitosan to 90mg of particles suspended in 1 ml of water. This mixture was placed in a bath-sonicator for 5 minutes before being shaken for one hour and finally freeze-dried. The fourth batch remained unmodified.

Saline solution (0.5ml) containing 100Lf diptheria toxoid was added to 10mg of the particles. Resulting suspensions were shaken overnight to enable adsorption of the antigen to the particle surfaces. following incubation, particles were washed by centrifugation with distilled water, as described above. In all cases, the antigen loading was determined to be in the region of 0.5% (w/w), giving an encapsulation efficiency of generally 50-60%.

Particles were fully characterised and mean particle diameters were found to be around 340nm for all batches.

Groups of Balb/c mice (n=5 per group) were dosed intranasally with the four freshly prepared suspensions, with doses equivalent to 5Lf units of DT per mouse, and a dosing volume of 20µl. Animals were bled periodically and ELISAs carried out to determine serum levels of anti-DT specific IgG titres.

The results for blood collected on Day 28 are shown in Figure 4.

Example 6

Immune responses to intramuscularly delivered diptheria toxoid (DT)

Eight formulations of pluronic/chitosan nanoparticles were prepared, by a simple sonication method. Deacetylated high molecular weight chitosan was obtained from Fluka. Three process variables were : type of Pluronic used (P101, P121 obtained from ICI Limited, UK), volume of pluronic added (75µl or 200µl per 2ml water), and addition or omission of chitosan. Briefly, to 2ml of double-distilled water, a small volume (75µl or 200µl) of the appropriate pluronic liquid (P101 or P121) was added. Mixtures were vortexed for one minute and sonicated for a further one minute. In order to coating the pluronic particles with chitosan, 100µl of a solution of 0.1% w/v high

molecular weight chitosan in 2% w/v glacial acetic acid was added to 100µl of each formulation. Finally, the diphtheria toxoid (DT) was adsorbed to the 200µl of coated and non-coated particles by the addition of 12µl of a solution of DT in water (4450lf units per ml). This preparation is a colloidal dispersion in water with a mean particle diameter of generally between 100-600nm. An example of a typical photon correlation spectroscopy (PCS) printout is shown in Figure 5.

Following characterisation of the prepared particulate formulations, groups of four or five female Balb/c mice were given a single dose of 50µl intramuscularly. The total equivocal dose for each animal was 5Lf units of DT. The final concentration of pluronics in the dosing medium was 5% (v/v) and chitosan 0.05% (w/v). Animals were bled periodically and ELISAs carried out to determine serum levels of anti-DT specific IgG titres.

Mean serum anti-DT IgG titres are shown herewith in Figure 6. It is clear from these results, that pluronics produce an enhanced immune response, either alone or in combination with chitosan.

Claims

1. A polycationic carbohydrate or a pharmaceutically acceptable derivative thereof, for use as an immunostimulant.

2. A polycationic carbohydrate according to claim 1 which is an immunostimulant comprising a chitin derivative, cationic polypeptide, cationic polyamino acid, a quaternary ammonium compound or a mixture thereof.

3. A polycationic carbohydrate according to claim 1 or claim 2 which comprises a chitin derivative.

4. A polycationic carbohydrate according to claim 3 wherein the chitin derivative is chitosan.

5. A polycationic carbohydrate according to claim 3 or claim 4 where the polycationic carbohydrate is a water-soluble chitin derivative.

6. A polycationic carbohydrate according to claim 5 wherein said water-soluble chitin derivative is an alkylated chitosan derivative or a salt thereof.

7. A polycationic carbohydrate according to claim 6 in which the alkylated chitosan is a trimethylchitosan.

8. A pharmaceutical composition comprising a biologically active agent which is capable of generating a protective immune response in an animal, and a polycationic carbohydrate according to any one of claims 1 to 7.

9. A pharmaceutical composition according to claim 8 which further comprises a diluent or carrier.

10. A pharmaceutical composition according to claim 9 which comprises particles comprising

(i) a biologically active agent which is able to produce an immune response in an animal to which it is administered;

(ii) a first material capable of forming particles; and

(iii) a polycationic carbohydrate according to any one of claims 1 to 6.

11. A composition according to claim 10 wherein the particle comprises microspheres, microparticles or liposomes.

12. A composition according to claim 10 wherein the particle comprises a microparticle.

13. A composition according to any one of claims 10 to 12 wherein the first material is a polymeric material which has a molecular weight of 100kDa or more.

14. A composition according to any one of claims 10 to 13 wherein the first material comprises poly-(L-lactide).

15. A composition according to any one of claims 10 to 14 wherein the ratio of the first material to the polycationic carbohydrate is from 99:1 to 9:1 w/w.

16. A composition according to any one of claims 8 to 15 wherein the biologically active agent is capable of generating a protective immune response against tetanus, diptheria, or *Yersinia pestis*.

17. A composition according to claim 16 wherein the biologically active agent comprises a combination of the V antigen of *Y. pestis* or an immunologically active fragment thereof, and the F1 antigen of *Y. pestis* or an immunologically active fragment thereof.

18. A composition according to any one of claims 8 to 17 which is adapted for intranasal application.

19. A composition according to any one of claims 8 to 18 which is adapted for parenteral administration.

20. A composition according to any one of claims 8 to 19 which further comprises an chemical compound selected from

A) a polyamino acid,
B) a vitamin or vitamin derivative,
C) cationic pluronics,
D) a clathrate,
E) a complexing agent,
F) cetrimides;
G) an S-layer protein; or
H) methyl-glucamine.

21. A composition according to claim 10 which further comprises a cationic pluronic.

22. A composition accoriding to claim 21 which comprises nanospheres of a cationic pluronic which are surface modified with chitosan.

23. A method for producing a pharmaceutical composition, which method comprises encapsulating a biologically active agent in a first material, in the presence of a polycationic carbohydrate according to any one of claims 1 to 6.

24. A method for producing a pharmaceutical composition which method comprises forming a microsphere, depositing a layer of polycationic carbohydrate according to any one of claims 1 to 6 thereon, and thereafter adsorbing a biologically active agent.

25. A method of protecting an animal against a pathogen, said method comprising administering to said animal, a protective

agent which is able to stimulate the animal's immune system to produce a response which is protective against said pathogen, and an immunostimulant comprising a polycationic carbohydrate.

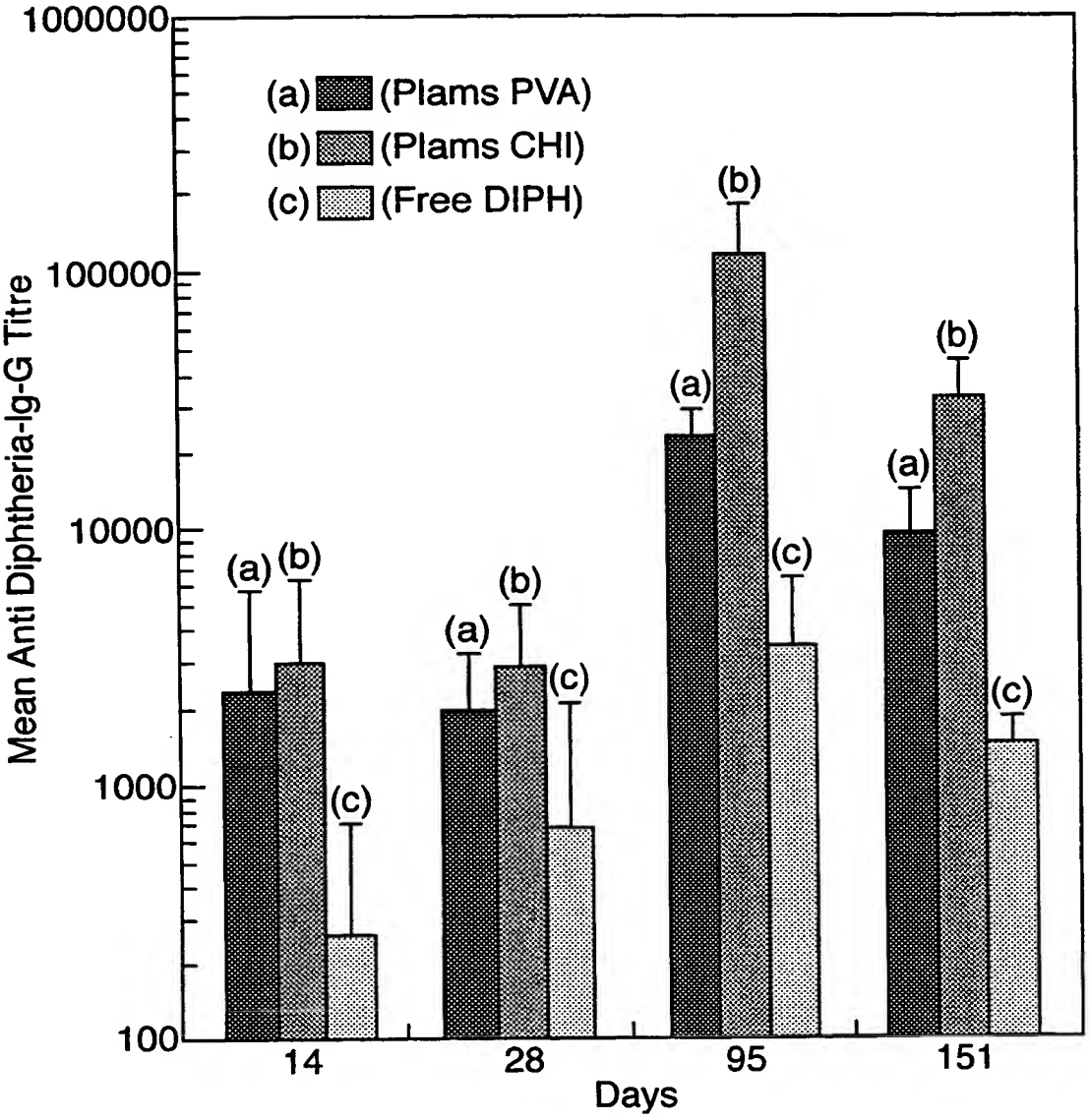
26. A method according to claim 25 wherein the protective agent which is able to stimulate the animal's immune system to produce a response which is protective against said pathogen, and an immunostimulant comprising a polycationic carbohydrate is applied parenterally or to a mucosal surface.

27. A method according to claim 26 wherein the protective agent and the immunostimulant are applied to a mucosal surface.

28. A method according to claim 27 wherein said mucosal surface is an intranasal surface.

29. The use of a polycationic carbohydrate or a pharmaceutically acceptable derivative thereof as an immunostimulant, in the preparation of a vaccine for use in prophylactic or therapeutic treatment.

Fig.1.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10000
1000
100
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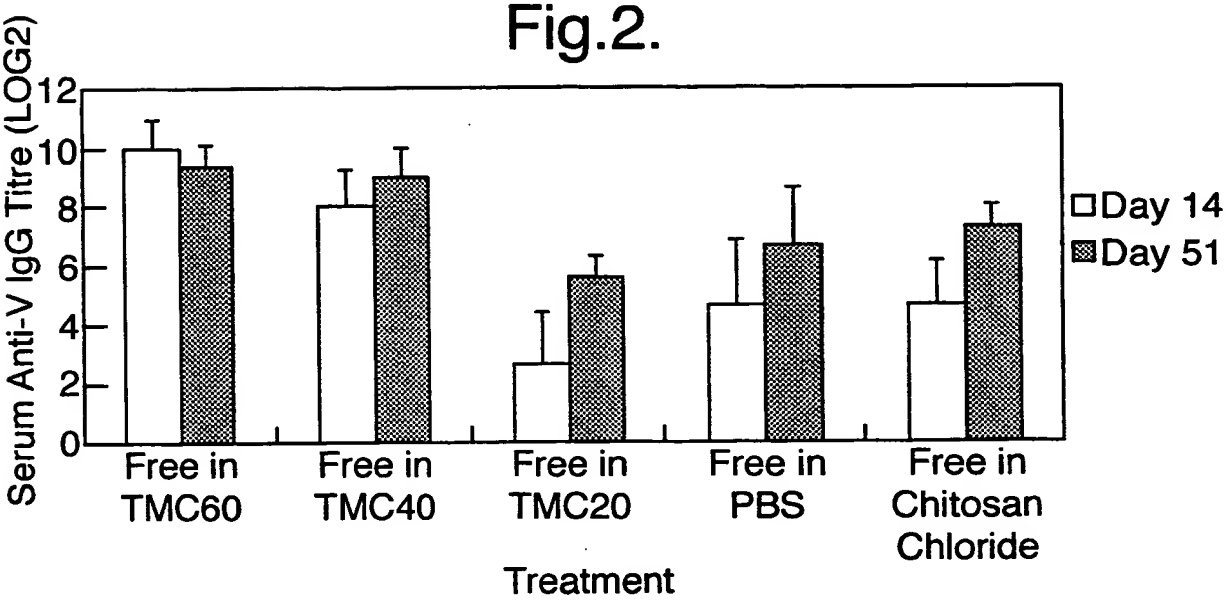
(a) (Plams PVA)
(b) (Plams CHI)
(c) (Free DIPH)
(a)
(b)
(c)
14
28
95
151
Days

Fig.2.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10
8
6
4
2
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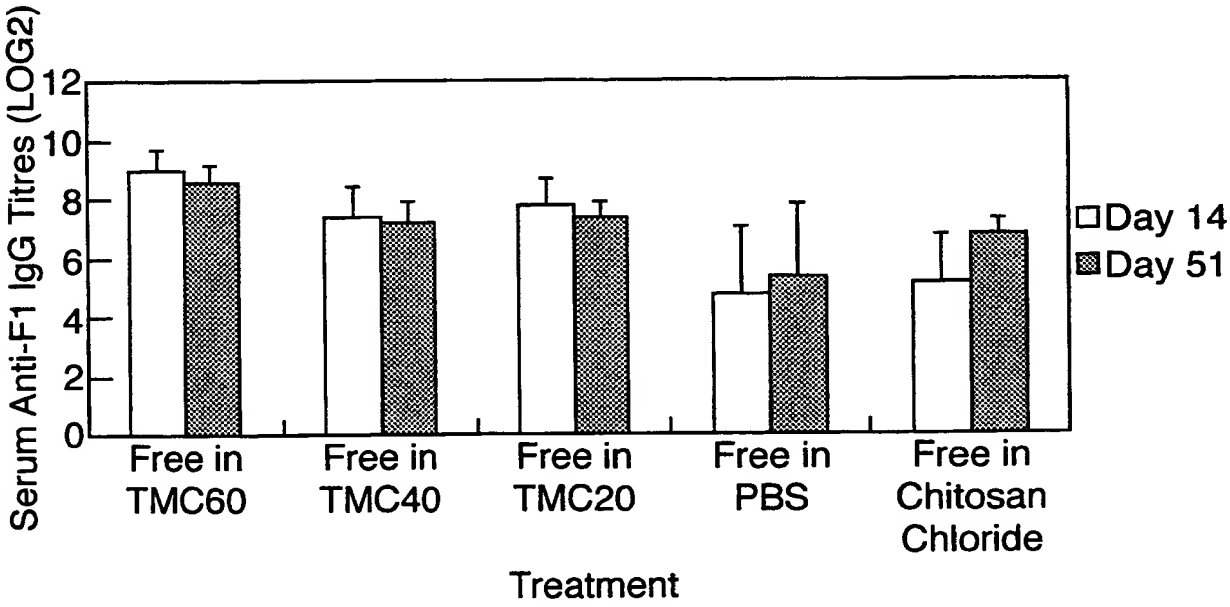
Free in TMC40
Free in TMC20
Free in PBS
Free in Chitosan Chloride
Treatment
Day 14
Day 51


Serum Anti-F1 IgG Titres (LOG2)
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10
8
6
4
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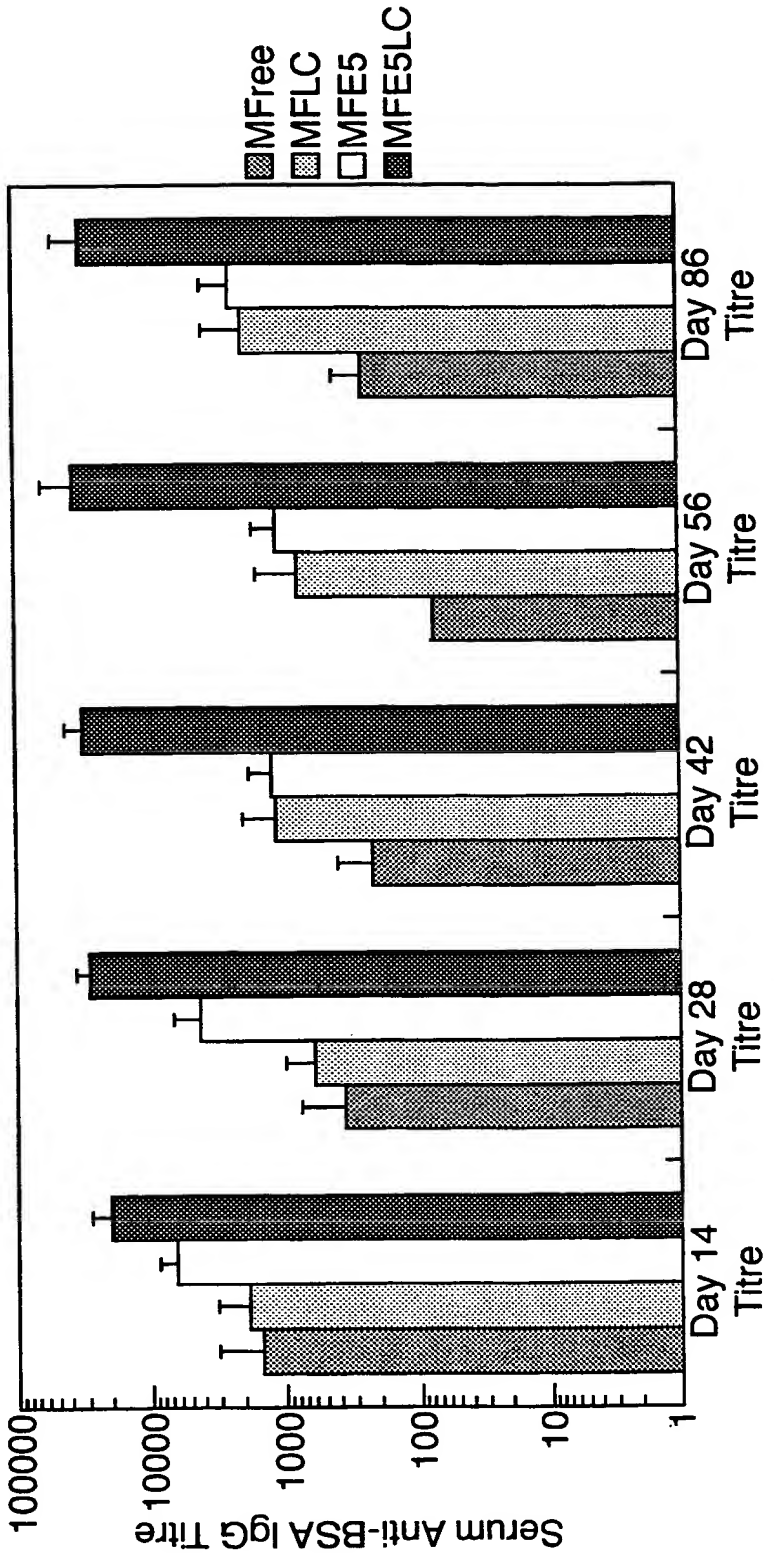
Free in TMC60
Free in TMC40
Free in TMC20
Free in PBS
Free in Chitosan Chloride
Treatment
Day 14
Day 51

Fig.3.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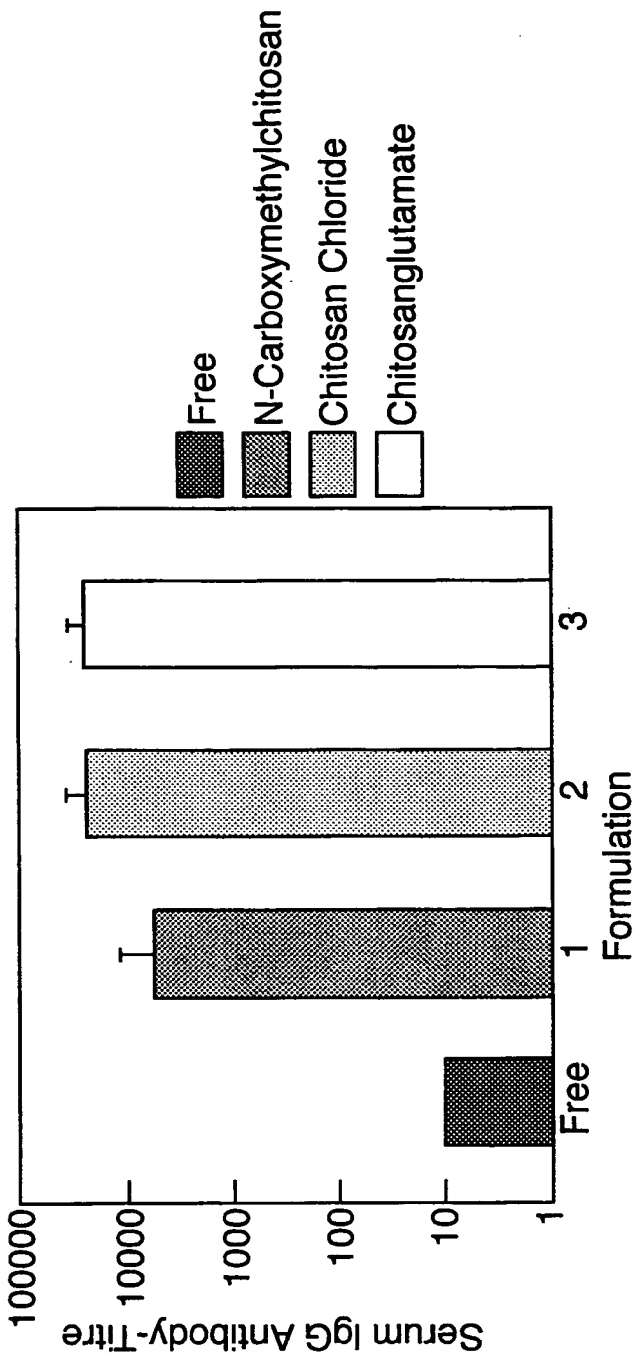
MFLC
MFE5
MFE5LC
Day 86 Titre
Day 56 Titre
Day 42 Titre
Day 28 Titre
Day 14 Titre
100000
10000
1000
100
10
1
Serum Anti-BSA IgG Titre

Fig.4.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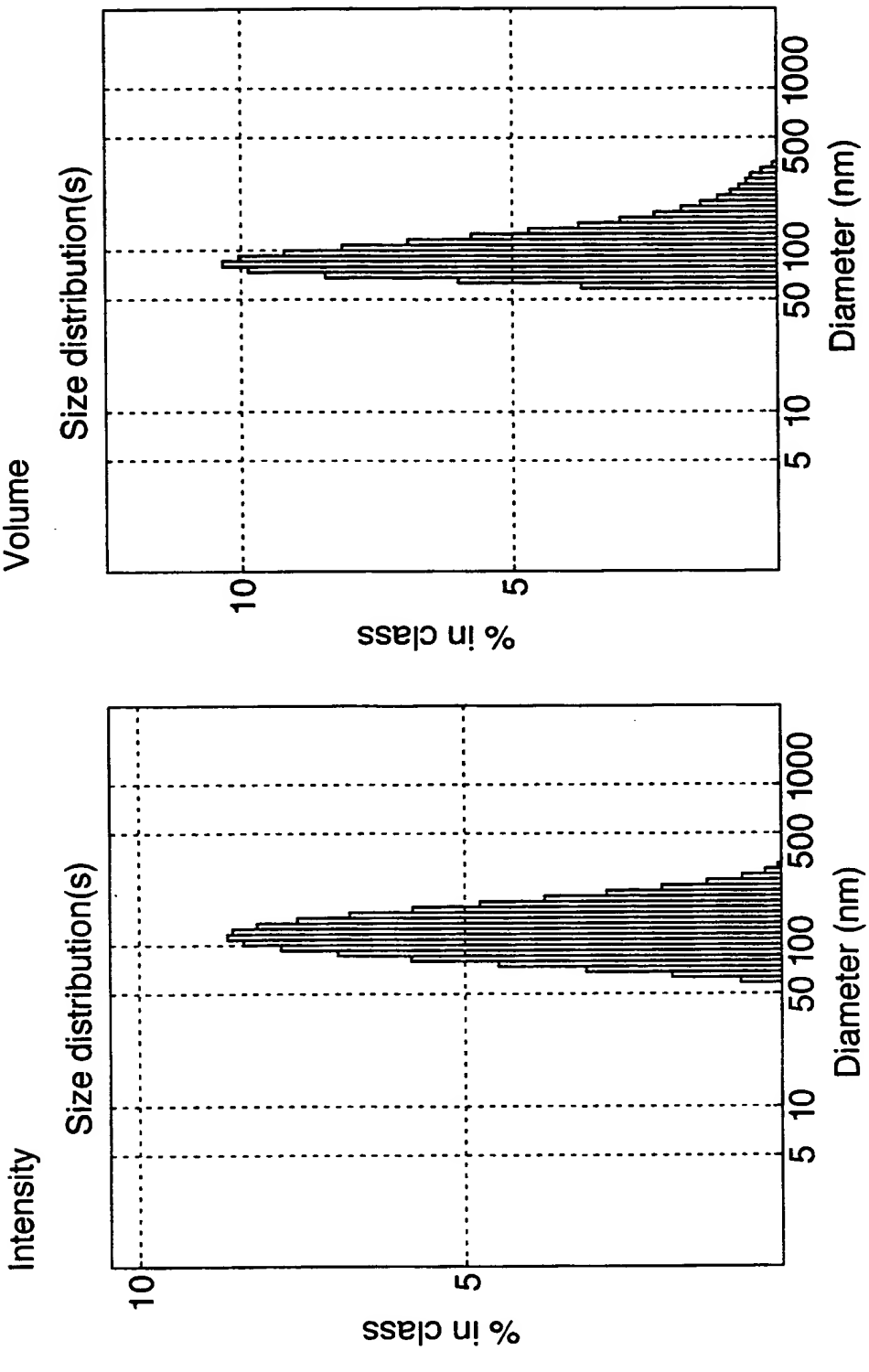
Chitosan Chloride
Chitosanglutamate
Serum IgG Antibody-Titre
100000
10000
1000
100
10
1
Free
1
2
3
Formulation

Fig.5.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5
Diameter (nm)
5 10 50 100 500 1000
Intensity
Size distribution(s)
% in class
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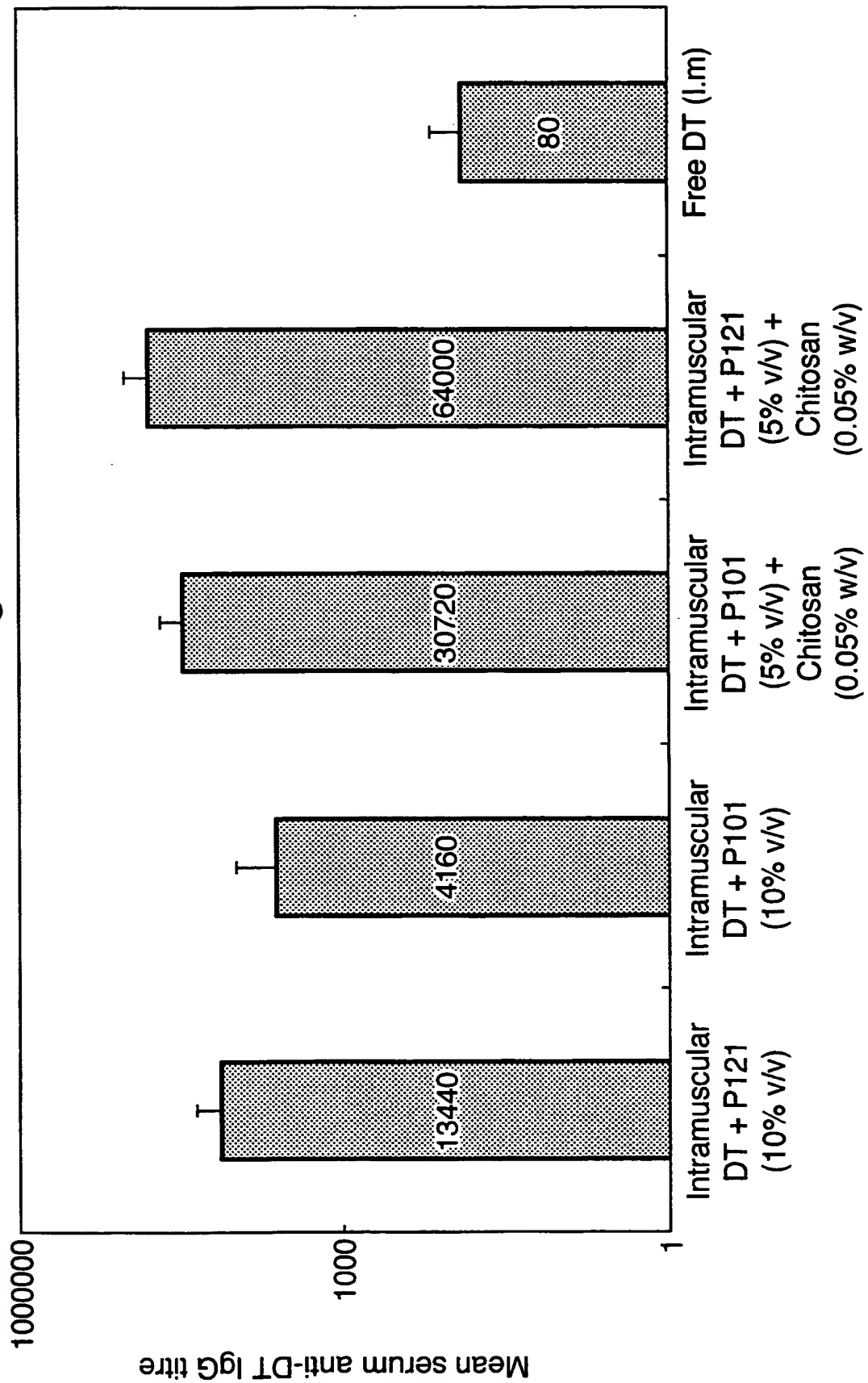
5
Diameter (nm)
5 10 50 100 500 1000

Fig.6.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4160
30720
64000
80
Intramuscular DT + P121 (10% v/v)
Intramuscular DT + P101 (10% v/v)
Intramuscular DT + P101 (5% v/v) + Chitosan (0.05% w/v)
Intramuscular DT + P121 (5% v/v) + Chitosan (0.05% w/v)
Free DT (I.m)

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(74) Agent: **BOWDERY, A., O.**; D/IPR, Formalities Section, Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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WO 00/56362 A3

(54) Title: POLYCATIONIC CARBOHYDRATES AS IMMUNOSTIMULANTS IN VACCINES

(57) Abstract: A polycationic carbohydrate such as chitosan, or a pharmaceutically acceptable derivative thereof, are used as immunostimulants. Vaccine compositions containing these polycationic carbohydrates, in particular in particles such as microparticles or liposomes are also described and claimed. Methods of treatment and the use of the polycationic carbohydrates as immunostimulants in the production of vaccines are further aspects described and claimed.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 00/01118

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/39 A61K39/02 A61K39/05 A61K9/127 A61K9/50
A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, PAJ, WPI Data, MEDLINE, EMBASE, LIFESCIENCES, AIDSLINE, CANCERLIT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 20576 A (DANBIOSYST) 12 June 1997 (1997-06-12) page 27 -page 30	1-9, 23-29
X	WO 96 10421 A (MEDEVA HOLDINGS) 11 April 1996 (1996-04-11) page 17 -page 19	1-9, 23-29
A	US 5 585 106 A (GRISTINA A.G. ET AL.) 17 December 1996 (1996-12-17) the whole document	1-29

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 October 2000	24/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KOTZE AWIE F ET AL: "Enhancement of paracellular drug transport with highly quaternized N-trimethyl chitosan chloride in neutral environments: In vitro evaluation in intestinal epithelial cells (Caco-2)." JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 88, no. 2, February 1999 (1999-02), pages 253-257, XP000792263 ISSN: 0022-3549 the whole document	1-29

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter...nal Application No
PCT/GB 00/01118

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